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Research paper

Use of bifunctional hybrid β-lactamases for epitope mapping and immunoassay development

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Abstract

Mapping of epitopes is a crucial step for the study of immune pathways, the engineering of vaccines and the development of immunoassays. In this work, the *Bacillus licheniformis* β-lactamase *BlaP* has been engineered to display heterologous polypeptides in a permissive and solvent-exposed loop. When combined with phage display, this modified enzyme can be used for epitope mapping by cloning random gene fragments. The proceedure presented in this paper allows the selection of large infectious phage libraries with high diversity and effieient β-lactamase activities. A useful aspect of the proposed teehnique results from the possibility of using the β-laetamase activity carried by phages to evaluate the proportion of immobilised phages during the successive enrichment steps of the library or competition experiments with the selected phages. Another advantage of the teehnique derives from the fact that the epitope is selected as a bifunctional hybrid protein, which can be overprodueed and purified. The resulting reeombinant protein associates an epitope with a specifie and effieient enzymatic activity. This constitutes an original tool for immunoassay development. A virus influenza hemagglutinin (*HA*1)-gene fragment library has been generated with this system and used to identify a linear epitope.

Keywords: Phage display; Epitope mapping; Hybrid proteins; β-Laetamase

1. Introduction

Phage display is a powerful technology to study protein/ligand interactions. According to this technique, peptides, proteins or protein fragments are expressed at the surface of phage particles in fusion with one of their coat proteins (Smith, 1985; Rodi and Makowski, 1999). The advantage of this system rests with the physical link between the heterologous polypeptide expressed at the surface of the phage and the corresponding nucleotide sequence cloned into the phagemid genome. The ability of each phage to replicate individually and to display a specific polypeptide on its surface permits high-throughput screening of libraries of variant nucleotide sequences with diversities up to 10^6 to 10^{10}. As a consequence, phage display has contributed to major advances in immunology, cell biology, drug discovery and pharmacology (Clackson et al., 1991; Lowman and Wells, 1993; Willats, 2002; Wang and Yu, 2004).

Presently, various phage display strategies have been developed to simplify epitope-mapping. These strategies are based on the display of random peptides (Smith

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Table 1
Sequence of the oligonucleotides used in this study

Oligonucleotides	Nucleotidic sequences (5′–3′)
*Bla*PApaI	AGTGCACAGTCTAACAATCAAACCAATGCCTCGCAACCT
*Bla*PNotI	TCTGCGGCCGCACGCCCCTCAATCCCTTTGCCGTTCATGTTTAAGGCTTTCAT
*Bla*PSacI	GAGCTCAGGCCTCTAACAATCAAACGAATG
*Bla*PSma−	CCCGGGATCTTCAAGTGCAAAGGCTCGAAGGCT
*Bla*PSma+	CTTGAAGATCCCGGGAAACTTCCAAGTGAAAAACGCGAG
*Bla*PEcoR1	GAATTCTTATTTTCCGTTCATGTTTAAGGCTTT
HA1+	CAAGACCTTCCAGGAAATGACAACTCGACAGCAACGCTGTGTCTGGGACAT
HA1−	AGTTTGTTTCTCTGGTACATTCCGCAT
epi*HA*	TATCCGTACGACGTACCAGATTACGCG

and Scott, 1993; Zwick et al., 1998) or DnaseI-generated overlapping gene fragments (Van Zonneveld et al., 1995; Wang et al., 1995) and the subsequent affinity selection on immobilised monoclonal (Scott and Smith, 1990) or polyclonal antibodies (Williams et al., 1998). In the case of random peptides, phage display leads to the selection of peptides which can be epitopes from the natural protein or mimotopes–peptides that bind tightly to the screening antibodies but bear no obvious identity to the native epitopes (Rodi and Makowski, 1999). Consequently, the assignment of the native epitope in the primary and/or tertiary structures of the antigen can be problematic and additional convergent evolutionary studies must be performed to unravel the protein interaction network. The phage display of DnaseI-generated overlapping gene fragments constitutes a good alternative to the display of random peptides because it refers directly to the natural amino acid sequence of the antigen. Gupta et al. (1999) have described a simple and efficient system for epitope mapping by cloning blunt-ended random gene fragments into an especially designed gIIIp-based phage display vector.

Another interesting approach has been described by Legendre et al. (1999). These authors describe the utilisation of the class A *TEM*-1 β-lactamase as a scaffold for protein recognition and assay. They identified two loops near the active site of the enzyme in which small peptides (six amino acids) could be inserted, replacing one or three codons of the *TEM*-1 gene (Legendre et al., 2002). Combination of mutated loops was also tested to increase the diversity of the library. In this way, they displayed libraries of hybrid β-lactamases and selected them for affinity towards specific targets. Allosteric regulations (inhibition or activation) of the enzymatic activity were observed for some of the hybrid proteins upon interaction with their specific targets (Legendre et al., 1999). In this application, several steps of affinity maturation were required to lower the dissociation constants into the micromolar to nanomolar range. Maturation was performed both on the inserted peptide(s) and the scaffold protein. The small size of the inserted peptides (six amino acids) and the bias favouring hybrid β-lactamases, which keep their enzymatic properties, might reduce the chance to map native epitopes. In the case of conformational linear epitopes, this implies that the β-lactamase constitutes a good scaffold which can display the random peptide in the native epitope conformation. For non-linear epitopes, the procedure of Legendre et al. (1999) offers the possibility of combining libraries in which random peptides are displayed in two different loops of the *TEM*-1 β-lactamase. Nevertheless, this approach suffers from the same drawbacks as all phage display procedures of random peptides in the assignment of amino acids that are involved in the interaction with the antibody.On the basis of their structures and mechanisms, four molecular classes (classes A, B, C, and D) of β-lactamases are recognised; three of these (classes A, C, and D) comprise active-site serine enzymes, and one (class B) is composed of zinc-dependent ("EDTA-inhibited") enzymes (Matagne et al., 1999). Class A(β-lactamases share similar 3D structures characterised by all α and α/β domains (Fonze et al., 1995, 2002). *Bla*P is a natural protease-sinsensitive class A β-lactamase that has been isolated from *Bacillus licheniformis*, a gram-positive bacterium that secretes various and numerous proteases (Filée et al., 2002; Brans et al., 2004; Veith et al., 1994). Attempts to express *TEM*-1 in different prokaryotes have been reported in the literature. These studies indicate that expression of *TEM*-1 often failed due to a high sensitivity to proteases (Wu et al., 1991). In contrast to *TEM*-1, *Bla*P can be overexpressed in many different microorganisms such as *E. coli*, *B. subtilis* and *Streptomyces*.

Previously, we have identified a permissive surface loop in *TEM*-1 which is located between α-helices 8 and

9 far from the active site (Fig. 1) (Ruth et al., 2005). This loop can accommodate long polypeptides (up to several hundred amino acids). The resistance to β-lactams is used to select bacteria that express functional hybrid β-lactamases. According to our data, expression assays in *E. coli* protease-deficient strains reveal that some insertions into *TEM*-1 are non-permissive not because of a misfolding of the β-lactamase but due to the increasing sensitivity of the hybrid proteins to proteases. It is suggested that the insertion of polypeptides induces some minor conformational changes, which expose embedded protein regions to proteases. This contributes to reduce the production yield of hybrid proteins and to limit the diversity of polypeptides that can be displayed in the β-lactamase. In this study, we have inserted blunt-end gene fragments in the DNA sequence encoding the *TEM*-1 corresponding loop of the protease-resistant class A *Bla*P β-lactamase. By using phage display, we have evaluated this approach for making a virus

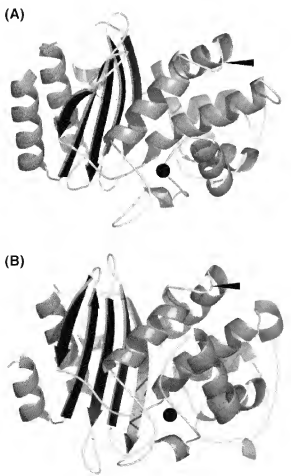

Fig. 1. Tertiary structures of the *TEM*-1 (A) and *Bla*P (B) β-lactamases. ●, catalytic site of the enzyme; and ▼, permissive insertion site used in this study.

influenza hemagglutinin (*HA*1) gene fragment library and selecting an epitope recognised by a high affinity monoclonal antibody against *HA*1.

The results show that a procedure alternating successive affinity selections of phages and growth of phage-infected cells in the presence of β-lactams permits the selection of bifunctional hybrid β-lactamases that present high affinity for the target ligand and good enzymatic parameters. We also show that these hybrid proteins can be successfully implemented in immunoassays.

2. Materials and methods

2.1. Construction of epitope display phage fdTet BlaP/SmaI

The gene encoding *Bla*P/*Sma*I was PCR amplified in a three-step procedure from genomic DNA of *Bacillus licheniformis* with primers *Bla*PApaI, *Bla*PSma−, *Bla*PSma+ and *Bla*PNotI (Table 1). Primers *Bla*PApaI and *Bla*PNotI created restriction sites suitable for cloning in fd-Tet-Dog1. Primer *Bla*PNotI also introduced a heptapeptide (Gly-Ile-Glu-Gly-Arg-Ala-Ala-Ala) between the C-terminus of *Bla*P/*Sma*I and the N-terminus of pIII. This heptapeptide corresponds to a Factor Xa cleavage site that can be used to elute the phages from the solid phase during affinity capture. The final PCR product was cloned into pGem T-easy and the nucleotidic sequence was verified by dideoxy sequencing. The gene encoding *Bla*P/*Sma*I was restricted by ApaII and NotI and subcloned in the corresponding sites of fd-Tet-Dog1 (Clackson et al., 1991) to yield fdtet *Bla*P/*Sma*I.

2.2. Preparation of HA1 gene fragments

This procedure was performed as described by Gupta et al. (1999). Briefly, the *HA*1 coding sequence was PCR-amplified using Taq DNA polymerase from a pGem T-easy *HA*1 plasmid with primers *HA*1+ and *HA*1− and purified on a GFX™ gel band purification kit (Amersham). The DNase shotgun cleavage kit (Novagen) was used to produce overlapping gene fragments in the range of 50–300 bp. The gene fragments were successively purified on a QIAquick™ nucleotide removal kit (Qiagen), repaired using Pfu polymerase (Promega) and purified again on a QIAquick™ nucleotide removal kit. The end-repaired gene fragments were dephosphorylated using calf intestine phosphatase (Boehringer) and purified on a QIAquick™ nucleotide removal kit.

2.3. Construction of fdtet BlaP/HA1 gene fragments library

The *HA*1 gene fragments were ligated in the *Sma*I-digested fdtet *Bla*P/*Sma*I using reagents from the pCR-Script Cloning Kit (Stratagene) except that *Srf*1 was replaced by *Sma*I. The ligation was carried out overnight at 18 °C. Enzymes were inactivated at 65 °C for 10 min. The ligation product was dialysed against water and used for electroporation in *E. coli* TG1 (Stratagene). Electro-transformed cells were regenerated in SOC medium for 1 h at 37 °C and plated on LB agar medium supplemented with 7.5 μg ml^{-1} tetracycline and 10 μg ml^{-1} ampicillin.

2.4. Phage preparation

The transformants were scraped and suspended in LB liquid medium supplemented with 7.5 μg ml^{-1} tetracycline and 10 μg ml^{-1} ampicillin. Phages were amplified at 28 °C for 16 h and recovered from the culture supernatant by two polyethylene glycol precipitations. After the first precipitation, filtration through a 0.45 μm filter was performed to eliminate membrane fragments. Phages were suspended in TBS (50 mM Tris, 150 mM NaCl at pH 7.4) and the phage concentration was estimated by measuring the absorbance at 265 nm.

2.5. Affinity selection

Biopanning was realised in Maxisorp® microplate strips (Nalge Nunc International). One microgram of rat monoclonal anti-*HA* (Roche) diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6) was coated per well for 16 h at 4 °C. After washing with TTBS (TBS with 0.5% Tween 20), wells were blocked with 10 mg ml^{-1} BSA in TTBS for 1 h at 37 °C. Then 10^{12} phages (100 μl of 1 nm OD_{265}) were added per well and incubated overnight at 4 °C. Unbound phages were removed by successive washings with TTBS and bound phages were eluted in 25 mM glycine–HCl buffer (pH 2.2) for 5 min. The eluate was transferred to a tube and neutralised with 2 M Tris–HCl (pH 8). The eluted phages were amplified by infecting log phase *E. coli* TG1. The phage titre was determined as tet^R and amp^R transduction units (TU) using TG1.

2.6. Western blot analysis

Phage proteins were denatured at 100 °C in the presence of denaturating buffer and separated by SDS–PAGE (10%). The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane. Immunoblot analysis using polyclonal anti-*Bla*P rabbit antibodies and detection of rabbit antibodies on blots were carried out using goat alkaline phosphatase-conjugated anti-rabbit antibodies (BioRad) and a colour reaction with 5-bromo-4-chloro-3-indoyl phosphate and nitro-blue tetrazolium, as instructed by the manufacturer.

2.7. PCR screening

The individual colonies were checked by PCR for the presence of a cloned *HA*1 gene-fragment into the *Bla*P gene (primers *Bla*PSmaseq and *BlaPEco*RI). PCR fragments larger than 400 bp confirmed the presence of an insert in *Bla*P. A second PCR was done to verify the presence of the nucleotidic sequence encoding the epitope recognised by the monoclonal anti-*HA* in the inserts (primer epi*HA* and *Bla*P*Eco*RI).

2.8. Phage ELISA

The analysis of the phages binding properties was performed as described for affinity selection except that elution was replaced by a revelation step. Two procedures were compared. The first uses a horseradish peroxidase conjugated anti-M13 MAb (Amersham) and the second takes advantage of the β-lactamase activity carried by the phages.

The anti-M13 monoclonal antibody was diluted 2500 times in TTBS supplemented with 10 mg ml^{-1} BSA and incubated in the wells for 1 h at 37 °C. Unbound monoclonal antibodies were removed by three washes with TTBS and one wash with TBS. ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid] (0.2 mg ml^{-1} final) in 50 mM citric acid (pH 4) was used as substrate to measure immobilised peroxidase activity at 405 nm.

The immobilised β-lactamase activity was measured by following the hydrolysis of 100 μM nitrocefin in 50 mM phosphate buffer pH 7.5 at 482 nm.

2.9. Cloning of hybrid β-lactamase encoding gene in pNY expression vector

The gene encoding the bifunctional hybrid protein of interest was PCR amplified from purified phagemide with primers *Bla*P*Sac*I and *Bla*P*Eco*RI. Primers *Bla*P *Sac*I and *Bla*P*Eco*RI create restriction sites suitable for cloning in pNY. Primer *Bla*P*Sac*I creates a restriction site that puts the gene of interest in frame with a sequence encoding a secretion signal sequence. Primer *Bla*P*Eco*RI also introduced a polyhistidine that could be used to purify the protein on Ni-PDC-4FF (Affiland). The final PCR product was cloned into pGem T-easy and the nucleotidic sequence verified by dideoxy

sequencing. The gene encoding the bifunctional hybrid protein was restricted by *Sac*I and *Eco*RI and subcloned in the corresponding sites of pNY. Plasmid pNY is an *E. coli* fully constitutive expression vector.

2.10. Bifunctional hybrid β-lactamase purification

To achieve production of the bifunctional hybrid proteins, *E. coli* JM109 transformed with pNY *Bla*P *HA* was grown in Terrific Broth supplemented with 75 μg ml^{-1} spectinomycin at 37 °C. Cells from an overnight culture (1 l) were harvested by centrifugation (9000 ×*g* for 15 min) and resuspended in 40 ml of TES (20% sucrose, 30 mM Tris–HCl, 5 mM EDTA, pH 8) at 37 °C. The bacterial suspension was stirred at 37 °C for 10 min. Cells were harvested by centrifugation (9000 ×*g* for 15 min) and the pellet resuspended in 40 ml of 5 mM $MgSO_4$ at 4 °C. The bacterial suspension was stirred at 4 °C for 10 min. The supernatant containing the periplasmic proteins was harvested by centrifugation (13,000 ×*g* for 20 min) and diluted with three volumes of 50 mM Tris–HCl (pH 7.5). The periplasmic proteins were loaded onto a Q-Sepharose Fast-Flow (Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.5). The proteins were eluted using a NaCl linear gradient (0–1 M) in 50 mM Tris–HCl (pH 7.5). The fractions containing the major β-lactamase activity were collected and pooled. In the second purification step, the pooled fractions were dialyzed against 50 mM phosphate (pH 8) at 4 °C and loaded onto a Ni-PDC-4FF column (Affiland) equilibrated in 50 mM phosphate (pH 8). The column was successively washed with 2 M NaCl and 50 mM phosphate (pH 8) supplemented with 10 mM imidazole. The bifunctional hybrid protein was eluted by an imidazole linear gradient (0–500 mM) in 50 mM phosphate (pH 8). Fractions containing the purified bifunctional hybrid proteins were pooled and dialyzed against PBS (50 mM phosphate, 150 mM NaCl, pH 7.4).

2.11. Titration and dissociation constant measurements

Titration assays were performed in Maxisorp® microplate strips (Nalge Nunc International). Increasing amounts of rat monoclonal anti-*HA* (Roche) diluted in carbonate buffer (50 mM $NaHCO_3$, pH 9.6) were coated for 16 h at 4 °C. After washing with TTBS (TBS with 0.5% Tween 20), wells were blocked with 10 mg ml^{-1} BSA in TTBS for 1 h at 37 °C. Bound monoclonal anti-*HA* was detected with 50 ng of purified *Bla*P *HA* in 10 mg ml^{-1} BSA in TTBS for 1 h at 37 °C. Unbound *Bla*P *HA* was removed by three washes with TTBS and one wash with TBS. The immobilised β-lactamase activity ($\Delta Abs/\Delta t$) was measured by following the hydrolysis of 100 μM nitrocefin in 50 mM phosphate buffer pH 7.5 at 482 nm. The dissociation constant (K_d) of the *Bla*P *HA*–anti-*HA* MAb complex was determined according to the ELISA protocol of Friguet et al. (1985). The *Bla*P *HA* concentrations that yielded a linear signal with 1 μg of coated anti-*HA* MAb were determined by following the immobilised β-lactamase activity as described above. A linear response for *Bla*P *HA* was obtained between 0 and 9.8 nM. In the assays, the percentage of *Bla*P *HA* bound to the immobilised anti-*HA* MAb was below 2.5% of the total amount of hybrid protein added in the well to avoid modification of the equilibrium in solution. To determine the K_d, various concentrations of free anti-*HA* MAb were preincubated with 4.89 nM of *Bla*P *HA*. After equilibrium was reached (1 h at room temperature), the concentration of free *BlaPHA* in each sample was determined with the help of anti-*HA* MAb-coated microplates as described above. The β-lactamase activities were plotted versus the concentration of free anti-*HA* MAb mixed with *BlaPHA*. The titration curve obeys the following equation:

$$A^2[E]_0 + A\left(A_0[L]_0 + A_0K_d - A_0[E]_0\right) - A_0^2K_d = 0$$

where $[E]_0$ is the initial concentrations of *Bla*P *HA*, $[L]_0$ is the total concentration of anti-*HA* MAb in solution, K_d is the dissociation constant, and A_0 and A are the activities measured on coated anti-*HA* MAb before or after preincubation with anti-*HA* MAb, respectively. The K_d was deduced by fitting the experimental points to the equation given above.

2.12. Kinetic studies

The kinetic parameters of the β-lactamase activity carried by phages and of the purified bifunctional hybrid β-lactamases were determined using nitrocefin in 50 mM phosphate buffer at pH 7. The initial rate of hydrolysis was monitored at 482 nm. The values of K_m and k_{cat} were calculated by fitting the data to the Henri–Michaelis equation and its linearised form according to the Hanes transformation.

2.13. ELISA

The ELISAs were realised using Maxisorp® microplate strips (Nalge Nunc International). Increasing amounts of purified *Bla*P/*Sma*I or *Bla*P *HA* diluted in carbonate buffer or PBS were coated for 16 h at 4 °C.

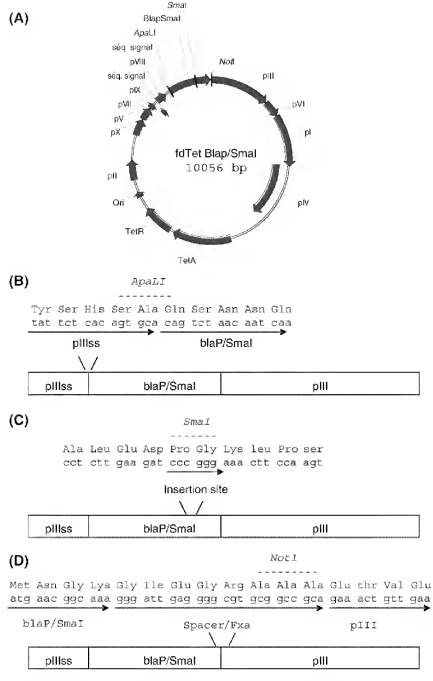

Fig. 2. Epitope display fd *BlaP*/*SmaI* phage. (A) Phagemide fd *BlaP*/*SmaI*; (B) nucleotidic sequence coding for the pIII signal sequence–*BlaP* junction; (C) nucleotidic sequence of the permissive insertion site engineered in *BlaP*; (D) nucleotide sequence coding for the *BlaP*–pIII junction. Fxa corresponds to the consensus peptidic sequence recognised by the Factor Xa protease.

The direct measurement of the immobilised β-lactamase activity after coating was done by following the hydrolysis of nitrocefin (100 μM) in 50 mM phosphate buffer (pH 7.5) at 482 nm after three washes of the plates with PBS.

For ELISA titration of coated β-lactamase, wells were blocked with 10 mg ml^{-1} BSA in PBS for 1 h at 37 °C. The horseradish peroxidase conjugated anti-*HA* MAb (Roche) was diluted 500 times in PBST (PBS 0.05% Tween 20) supplemented with 10 mg ml^{-1} BSA and incubated in the wells for 1 h at 37 °C. Unbound monoclonal antibodies were removed by three washes with PBST and one wash with PBS. Peroxidase activity was measured as above.

3. Results

3.1. Engineering of epitope display fdTet BlaP/SmaI phage

FdTet *Bla*P/*Sma*I phage carries the nucleotidic sequence coding for the engineered class A *Bla*P/*Sma*I β-lactamase that is displayed at the surface of phage particles in fusion with the minor coat pIII protein (Fig. 2). The gene encoding *Bla*P/*Sma*I was obtained by insertion of two codons (CCC-GGG), corresponding to a *Sma*I restriction site and coding for the dipeptide Pro-Gly, between residues Asp211 and Lys212 of *Bla*P β-lactamase.

E. coli TG1 transformed with fdTet *Bla*P/*Sma*I are resistant to tetracycline (7.5 μg ml^{-1}) but can also grow in the presence of ampicillin (10 μg ml^{-1}). These characteristics offer the opportunity of a positive selection of functional hybrid β-lactamases during the construction of phage libraries. Cell fractionation and extraction by cold osmotic shock confirmed the presence of β-lactamase activity in the periplasm, suggesting that the post-transcriptional maturation of the fusion *Bla*P/*Sma*I-pIII protein does not result in formation of inclusion bodies and bulking of the cytoplasmic membrane.

Western blot analysis (Fig. 3) and β-lactamase assays (data not shown) on purified phage particles confirmed the display of functional β-lactamases in fusion with pIII on the surface of the phages. *E. coli* TG1 infected with purified fdTet *Bla*P/*Sma*I phages grew in the presence of both tetracyline (7.5 μg ml^{-1}) and ampicillin (10 μg ml^{-1}). This demonstrates that the phage infectivity is not altered by the display of *Bla*P in fusion with pIII.

3.2. HA1 gene fragment library

The *HA*1 gene was PCR amplified both to obtain large quantities of template and to limit the source of contaminating DNA for the construction of the library.

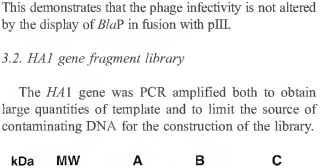

Fig. 3. Immunodetection of hybrid β-lactamases exposed at the surface of phage particles. The western blotting was performed with purified phages and rabbit anti-*Bla*P serum. Lanes A, B and C were obtained with phages which express *Bla*P without insert and *Bla*P with 150 bp and 300 bp long inserts, respectively. The apparent molecular masses of pIII and *Bla*P-pIII proteins are about 70 and 100 kDa, respectively.

Random DNase digestions were performed to produce *HA*1 gene fragments in the range of 50–300 bp. The fragments were successively end-repaired and dephosphorylated to prevent cloning of two or more fragments in the *Bla*P/*Sma*I gene. The ligation was done in the presence of *Sma*I to prevent self-ligation of the fdTet *Bla*P/*Sma*I vector.

A primary library of 4×10^5 transformants was obtained on LB agar supplemented with tetracycline (7.5 μg ml^{-1}) and ampicillin (10 μg ml^{-1}). A PCR analysis was performed on individual colonies to check the presence of *HA*1 gene fragments in the *Bla*P/*Sma*I gene. More than 95% of the tetracycline and ampicillin resistant clones were controlled as recombinants. The size distribution of the inserts was up to 300 bp. Western blot analysis confirmed the presence of hybrid β-lactamases fused to pIII at the surface of phages (Fig. 3). An infection step was performed to eliminate non-infectious phages. This was done by infecting *E. coli* TG1 with phages derived from the primary transformants. As previously, PCR analysis revealed that more than 95% of the clones were recombinants.

3.3. Epitope mapping

Phages derived from the second library were prepared and selected on immobilised anti-*HA* MAb according to the procedure described in Fig. 4. Eluted phages were used to infect *E. coli* TG1. Three rounds of affinity selection were performed. The PCR analysis indicates that 50% of the clones express the *HA* epitope after one round of affinity selection. This percentage reaches 100% after the second round of panning. The nucleotide sequences of the clones from the second round of panning are presented in Fig. 5. These confirm the presence of the epitope in protein fragments of variable sizes. The multiple alignment allows the epitope to be accurately positioned in the primary structure of *HA*1. The sequencing of the clones from the third round of panning indicates a much lesser diversity of the fragments harbouring the epitope (data not shown). In consequence, if the redundant fragment is too long, the epitope cannot be precisely assigned. For instance in Fig. 5, if additional panning round reduces the diversity to fragments 1–6, the consensus epitope will be 42 residues long rather than 12.

3.4. Use of the β-lactamase activity to monitor the enrichment steps of the library

In our procedure, enrichment of the library is not only based on the affinity of phages for antibodies but also

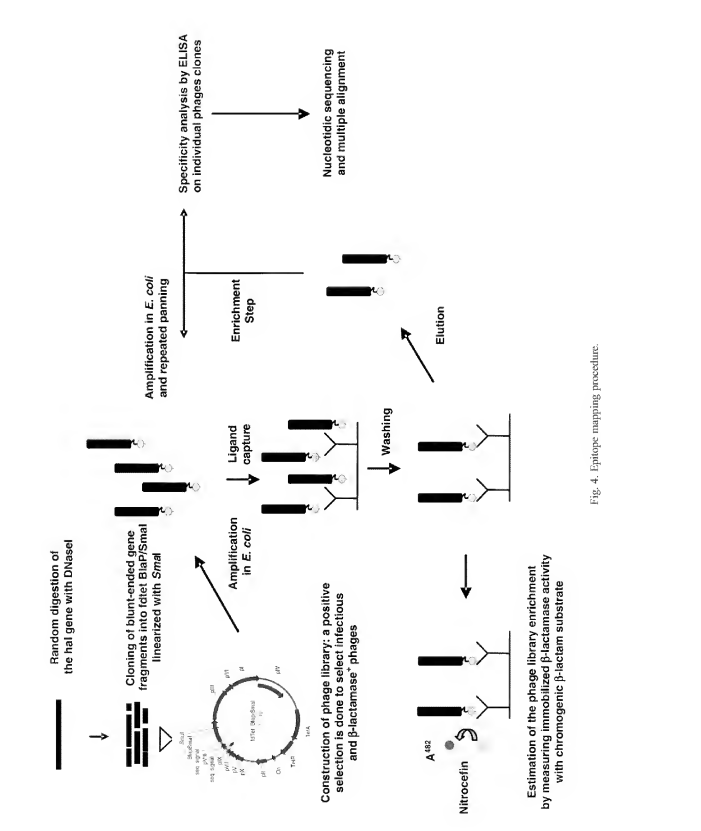

Fig. 4. Epitope mapping procedure.

(A)

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Influenza virus HA1

1       QDLPGNDNST ATLCLGHHAV PNGTLVKTIT NDQIEVTNAT ELVQSSSTGK ICNNPHRILD

51      GIDCTLIDAL LGDPHCDGFQ NETWDLFVER SKAFSNCYPY DVPDYASLRS LVASSGTLEF

101     ITEGFTWTEV TQNGGSNACK RGPGSGFFSR LNWLTKSGST YPVLNVTMPN NDNFDKLYIW

151     GVHHPSTNQE QTSLYVQASG RVTVSTRRSQ QTIIPNIGSR PWVRGQSSRI SIYWTIVKPG

201     IDVLVINSNGN LIAPRGYFKM RTGKSSIMRS DAPIDTCISE CITPNGSIPN DKPFQNVNKI

251     TYGACPKYVK QNTLKLATGM RNVPEKQT
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(B)

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Cl 1    FVERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 2     VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGS
Cl 3     VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGS
Cl 4     VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 5     VERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 6      ERSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGSNACKRGPGSGFFS
Cl 7       RSKAFSNCYPYDVPDYASL
Cl 8       RSKAFSNCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQNGGSNACKRGPGSGFFSRLNWLTKSEV
Cl 9             NCYPYDVPDYASLRSLVASSGTLEFITEGFTWTEVTQN
Cl 10              CYPYDVPDYASLRSLVASSGTLEFITEGFTWTEV
Cl 11             NCYPYDVPDYASL

Consensus          CYPYDVPDYASL
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Fig. 5. Epitope mapping results of anti-*HA* MAb. (A) Amino acid sequence of the *HA*1 fragment of the influenza virus. (B) Eleven individual clones from the second round of panning were sequenced. A multiple sequence alignment is presented. The residues corresponding to the epitope recognised by the anti-*HA* MAb are underlined.

involves a competition between phage-infected cells during the culture. During phage preparation, phage-infected cells are grown in a liquid medium supplemented with ampicillin. Under these conditions, the bacterial growth is directly associated with antibioresistance. As seen above, expression of the fusion *Bla*P/pIII protein confers resistance to β-lactams to infected cells.

Fig. 6 shows the immobilised β-lactamase activity on anti-*HA* MAb during the successive enrichment steps. These experiments indicate a strong increase of the immobilised β-lactamase activity after the first round of panning. According to PCR analysis as described above, this is due to the significant enrichment of the library in phages harbouring the good epitope. For the second and third rounds of panning, the immobilised β-lactamase activity continues to increase but more moderately. This can be partially explained by the fact that all the phages harbour the good epitope and that the two determining factors are the competition between phages for the binding to anti-*HA* MAb and the competition between phage-infected cells during growth in liquid medium. The sequencing of phages has revealed that the epitope was present in fragments of variable sizes (Fig. 5) and consequently with distinct folds and solvent accessibility. This could modulate the affinity of the anti-*HA* MAb for the hybrid β-lactamases and, in consequence, when

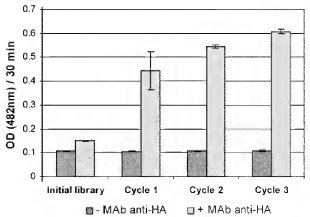

Fig. 6. Monitoring of the phage library enrichment. β-Lactamase assays were performed in the presence or in the absence of coated MAb anti-*HA* (1 μg). Nitrocefin (100 μM) was used as substrate. The same phage titre (7×10^{11} phages per well) was used in all the assays.

Table 2
Kinetic studies of phage libraries during the successive enrichment steps

Cycle	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
0	74±15	248±20	3.4±0.9
1	38±1	360±40	9.5±3
2	32±2	350±7	11±1
3	36±4	410±60	11±3
3	36±2	540±90	15±3

Three biopanning steps were realised. Phages were prepared from liquid culture medium supplemented with tetracycline (7.5 μg ml^{-1}) and ampicillin (10 μg ml^{-1}). The ampicillin concentration was fixed at 25 μg ml^{-1} in liquid culture medium used to prepare phages from cycle 3 and the selection of phage infected bacteria was realised on LB agar supplemented with tetracycline (7.5 μg ml^{-1}) and ampicillin (100 μg ml^{-1}). The substrate was nitrocefin.

the target ligand on the solid phase becomes the limiting factor, the phages are selected as a function of their relative affinities. Not unexpectedly, the display of random polypeptides in the *Bla*P β-lactamase generates hybrid proteins with different kinetic parameters. Under our selection conditions, we found that the catalytic efficiencies (k_{cat}/K_m) of the selected hybrid proteins were in the range of 3–28 μM^{-1} s^{-1} on nitrocefin. The growth of phage-infected bacteria in the presence of a β-lactam allows the selection of those transformants which secrete the most efficient hybrid β-lactamases. The data reported in Table 2 are in agreement with this hypothesis since the catalytic efficiencies (k_{cat}/K_m) of the phage libraries increase during successive rounds of panning. To calculate the global k_{cat} and K_m values of the different phages libraries, we assumed that each phage displays one molecule of hybrid β-lactamase. Moreover, it appears that the selection of very efficient hybrid proteins can be improved by increasing the β-lactam concentrations (100 μg ml^{-1}) in the culture medium when preparing the phage particles (25 μg ml^{-1}) and selecting phage-infected bacteria (100 μg ml^{-1}).

3.5. Comparison of phage ELISA methods

The reference method to measure the interaction between a phage and its target ligand is time-consuming because it is based on the use of horseradish peroxidase conjugated anti-pVIII MAb. This is not the case when using the intrinsic β-lactamase activity carried by the fdTet *Bla*P/*Sma*I phage. The length of this filamentous phage is about 1 μm and it contains a circular ssDNA wrapped inside an assemblage of 2670 copies of the major coat protein pVIII. The head region of the phage plays a crucial role for binding to the host cell. This region contains only five copies of pIII. Consequently, it is difficult to estimate the binding stoichiometry when using anti-pVIII MAb. The data presented in Fig. 7 show that the sensitivity levels of the β-lactamase and peroxidase assays are similar. As a negative control, the fdTet *Bla*P/*Sma*I phage without insert in the β-lactamase gene was used. In these experiments, increasing quantities of anti-*HA* MAb (0–200 ng) were coated on microplates in carbonate buffer. The experiments were performed with the same phage titres.

3.6. Implementation of purified bifunctional hybrid proteins in immunoassays

The gene coding for a hybrid β-lactamase displaying only the *HA* epitope sequence was subcloned in the full constitutive expression vector pNY. The protein was overproduced in *E. coli* JM109 and purified by affinity chromatography on Ni-PDC-4FF thanks to a

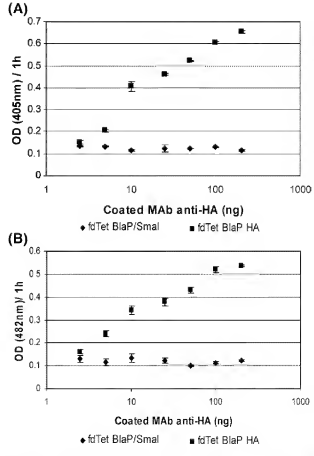

Fig. 7. Comparative analysis of the methods used to detect phages interactions. (A) The reference method which uses a horseradish peroxidase conjugated anti-PVIII MAb. (B) The method using the intrinsic β-lactamase activity carried by phages. The experiments were performed in the presence of increasing quantities of coated MAb anti-*HA*. All assays were performed with the same phage titre (7×10^{11} phages per well).

Table 3
Kinetic studies of hybrid β-lactamases

Enzymes	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)
BlaPHA (25 °C)	48±2	1350±50	28±1
BlaP SmaI (25 °C)	48±1	1260±140	26±2
BlaP WT (37 °C)	35	1800	51

BlaP SmaI and *BlaP HA* correspond to the engineered form of *BlaP* without insert and to a hybrid β-lactamase displaying only the sequence corresponding to the *HA* epitope. The substrate was nitrocefin. The values were compared to the data reported for the wild type enzyme *BlaP* WT (Matagne et al., 1990).

polyhistidine tag at the C-terminal end of *Bla*P. The K_m and k_{cat} values of the purified hybrid β-lactamase were similar to those of the native and *Bla*P/*Sma*I enzymes (Table 3). No significant change in the catalytic efficiency (k_{cat}/K_m) was observed upon antibody binding suggesting that the interaction does not induce significant conformation perturbation at the level of the active site (data not shown). The dissociation constant (K_d) of the *Bla*P *HA*–anti-*HA* MAb complex was deduced from ELISA titrations of unbound *Bla*P *HA* in the presence of increasing concentrations of anti-*HA* MAb (Fig. 8) yielding a K_d value of about 0.68 nM.

We have studied the possibility of using the β-lactamase as a carrier protein for displaying epitopes on a solid-phase for performing ELISAs (Fig. 9). The coating was done in PBS and the detection of the hybrid protein was performed with horseradish peroxidase conjugated anti-*HA* MAb. The titration curve clearly indicates the validity of such an approach. A good correlation is observed between the immobilised peroxidase activity and the amount of coated hybrid β-lactamase in the range from 5 to 100 ng. This suggests that the solvent accessibility of the *HA* epitope is not altered when the hybrid protein is coated. Another interesting aspect of the technique results from the fact that the β-lactamase activity can be measured to verify the coating efficiency and to quantify the coated protein. Fig. 10 compares the immobilised β-lactamase activities on microplates after coating of increasing concentrations of *BlaPHA* in phosphate buffer saline and carbonate buffer. These results indicate that the coating seems to be more efficient in phosphate buffered saline. In parallel, we performed a limited study on the human seroprevalence to the *Bla*P/*Sma*I β-lactamase. Fifteen sera were tested

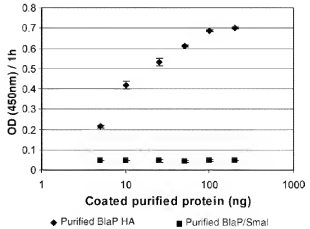

Fig. 9. Use of hybrid β-lactamase as a substitute for native antigen in immunoassays. Increasing quantities of purified *BlaP SmaI* and *BlaP HA* were coated on microplates. Horseradish peroxidase conjugated anti-*HA* MAb was used to titrate coated β-lactamases.

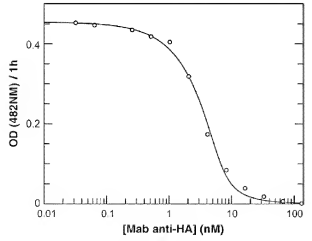

Fig. 8. Titration curve of free *Bla*P *HA* as a function of anti-*HA* MAb concentration. *BlaP HA* was preincubated with increasing concentrations of MAb anti-*HA*. The concentration of free *BlaP HA* at equilibrium was estimated on anti-*HA* MAb coated microplates by measuring the immobilised β-lactamase activity.

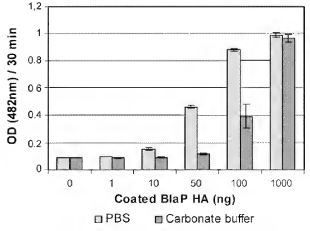

Fig. 10. Comparison of coating conditions. The efficiency of the coating (carbonate buffer or PBS) was compared by measuring the immobilised β-lactamase activity.

for the presence of anti-*Bla*P/*Sma*I IgG. No seroprevalence against the protein was observed (data not shown).

4. Discussion

In immunology, epitope mapping is used to study antigen/antibody (B-cell epitope) or antigen/T cell receptor (T cell epitope) interactions, develop immunoassays, identify neutralising sites, produce protective peptide vaccines, develop hypoallergens, investigate the pathogenesis of autoimmune diseases, etc. Moreover, all the epitope mapping techniques share a common characteristic: the epitope is always mapped in an unusable form which can not be directly used throughout the R and D process.

In this study, we have demonstrated that the hybrid β-lactamase technology, when coupled with phage display, not only identifies protein regions involved in protein–protein interactions but also supplies useful molecules where these regions are linked to an enzymatic activity. We have validated this approach for making an influenza virus hemagglutinin (*HA*1) gene fragment library and selecting a linear epitope recognised by a monoclonal antibody. The results have shown that a procedure alternating successive affinity selection of phages and growth of phage-infected cells in the presence of β-lactams permits the selection of bifunctional hybrid β-lactamases with high affinity for the target ligand and good kinetic parameters. We have demonstrated that modifying the β-lactam concentration in the culture medium allows a positive selection of hybrid β-lactamases that retain a native-like catalytic efficiency. This is a useful tool to accelerate the convergent selection of bifunctional β-lactamases.

PCR analysis and nucleotidic sequencing have revealed that the *Bla*P β-lactamase can display both short and large polypeptides belonging to the same protein domain. It is thus expected that that this region adopts different folds as a function of the inserted protein fragment. This constitutes an interesting feature for mapping non-linear epitopes. This work is currently underway.

Compared to classical phage display procedures, a useful aspect of the proposed technique results from the ability to use the β-lactamase activity carried by phages as a reporter enzyme to directly monitor specific phage interactions. This work shows that the selected bifunctional hybrid β-lactamases can be easily overproduced in *E. coli*, purified and utilised in immunoassays. Other applications can be envisaged. Indeed, bifunctional hybrid β-lactamases can be utilised in biosensor and affinity chromatography development, drug screening, drug targeting, vaccine development, and production and selection of hybridoma. In conclusion, hybrid β-lactamase technology offers new opportunities in epitope mapping and permits the development of new tools of interest for diverse applications.

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